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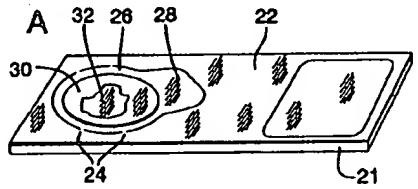
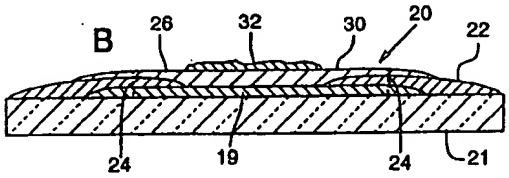
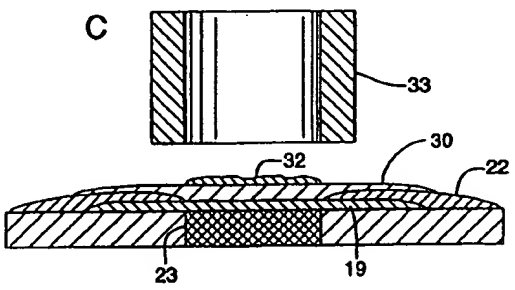
(54) Title: CYTOPERFUSION OF TISSUE SPECIMENS

(57) Abstract

A tissue specimen (32) is placed on a composite support (20) of nitrocellulose (30) and porous nylon (19) and lyophilized to produce vertical microchannels through the specimen (32). Liquid immunocytological reagents are then perfused through the porous specimen (32) and support (20), providing intimate contact of specimen (32) and reagents.







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CYTOPERFUSION OF TISSUE SPECIMENS

FIELD OF THE INVENTION

This invention concerns histological preparation and examination of tissue specimens for diagnostic purposes. In more particular embodiments, the invention is directed to cytologic and immunofluorescent examination of pathological specimens for diagnostic purposes.

BACKGROUND OF THE INVENTION

Microscopic examination of tissue specimens for histopathologic purposes has greatly enhanced the effectiveness of medical treatments for a variety of illnesses, because microscopic examination permits a more specific diagnosis of a pathological condition. In standard anatomical pathology, a diagnosis is made on the basis of cell morphology and staining characteristics. Tumor specimens, for example, can be examined to characterize the tumor type and predict whether the patient will respond to a particular form of chemotherapy. Although the microscopic examination and classification of tumors has improved medical treatment, the microscopic appearance of a tissue specimen stained by standard methods (such as hematoxylin and eosin) can only reveal a limited amount of diagnostic information.

Recent advances in molecular medicine have provided an even greater opportunity to understand the cellular mechanisms of disease, and select appropriate treatments with the greatest likelihood of success. Some hormone dependent breast tumor cells, for example, have an increased number of estrogen receptors on their cell surfaces that indicate that the patient from whom the tumor was taken will respond to certain anti-estrogenic drug treatments. Other diagnostic and prognostic cellular changes include the presence of tumor specific cell surface antigens (as in melanoma), the production of embryonic proteins (such as α-fetoprotein in liver cancer and carcinoembryonic glycoprotein antigen produced by gastrointestinal tumors), and chromosomal abnormalities (such as the presence of oncogenes). A variety of techniques have evolved to detect the presence of these cellular abnormalities, including immunophenotyping, in situ hybridization, flow cytometry, and DNA amplification using the polymerase chain reaction (PCR).

One approach to molecular diagnosis has been to homogenize cells from a tissue specimen, to expose the cellular contents to a liquid environment in which analysis of the biochemical markers can be performed. This approach has been used to detect changes in the concentration of epidermal growth factor (EGF) or estrogen receptors (ER) in breast cancer. A drawback of this method has been that it destroys the cytocoherence of the cells, which can remove important information about the tumor. Another problem with this quantitative assay is that malignant cells often proliferate dramatically, while the cell surface marker proliferation is more subtle. The greater proliferation of the cellular compartment can overwhelm the more subtle

increase in the cell surface markers, thereby obscuring quantitative information about marker concentration.

Another approach to molecular diagnosis of tissue specimens has been to expose the tissue to probes. A probe is any molecule that specifically binds to a nucleic acid sequence or protein of interest, and which can be labeled so that the required targets can be detected. Probes for nucleic acid sequences are radiolabeled or chemically tagged oligonucleotides of complementary sequence to the DNA or RNA sequence that is diagnostic of a genetic change associated with a tumor (such as the Philadelphia chromosome in chronic granulocytic leukemia). A monoclonal antibody can be designed to recognize antigens that are biochemical markers (such as the Her-2 and epidermal growth factor (EGF) receptors in breast cancer). The antibodies may undergo a chromogenic immunochemical reaction that is proportional to the concentration of the biochemical marker, such that the concentration of the marker on the cell can be colorometrically quantitated. The reaction may either be direct (fluorochrome directly conjugated to the primary antibody) or indirect (two-stage procedure in which a secondary fluorochrome conjugated antibody is directed against the primary antibody). Such basic techniques of immunohistochemistry (including immunofluorescent staining methods and enzyme conjugation procedures) are disclosed in DeLellis, Diagnostic Immunohistochemistry, Masson Publishing, 1981, chapter 1 and 2.

The use of immunohistochemical analysis, however, is limited by the techniques used to prepare tissue specimens. Standard preparation techniques usually require that a paraffin embedded or thin frozen section be mounted on a nonporous glass or plastic slide. The specimen is usually first chemically fixed with a cross-linking agent (such as formaldehyde), or embedded with resin, to preserve the specimen for prolonged storage. These manipulations harden the specimen, and make it difficult to introduce probes into it. Subsequent chemical treatments of the specimen may be used to soften or "unfix" it, which improves access to probes, but only at the expense of disrupting cellular relationships and degrading the image. "Unfixation" methods, such as enzyme digestion or antigen retrieval with heating and strong alkali are described in Taylor and Cote, Immunomicroscopy: A Diagnostic Tool for the Surgical Pathologist, 2nd ed., 1994, WB Saunders, chapter 3.

Another problem with fixation of tissue specimens on slides is that cellular solutes diffuse from the specimen during chemical fixation, and these solutes may be lost or transported away from their original locations. Solute diffusion can reduce the signal intensity of quantitative immunoassays, and degrade the quality of the magnified image. This problem has been addressed in PCT Publication No. WO 93/04193, which discloses that cytocoherent transfer of a tissue specimen can be achieved by thawing a frozen sample on a membrane impregnated with a surfactant. Cytocoherent transfer is a transfer that preserves the location of the target cell solute (such as the location of a protein receptor in the cell membrane) with accurate planar definition relative to cellular microanatomy and organelles with which the solute associates. The approach disclosed in this PCT publication is said to avoid channeling of the tissue specimen, which can result from ice

crystal formation and sublimation, and which causes undesirable dessication and deterioration of tissue that can occur with quench freezing of the sample. Cytocoherent transfer methods are also disclosed in McGrath et al., "High-Definition Cell Analysis In Situ Using Microporous Films", *Cell Vision*; 2:499-509 (1995), and McGrath et al., "Cytometrically Coherent Transfer of Receptor Proteins on Microporous Membranes", *BioTechniques*; 11:352-361 (1991).

Lyophilization of pathologic specimens for subsequent analysis has also been discussed in the scientific literature. An example is Stumpf and Roth, "High Resolution Autoradiography with Dry Mounted, Freeze-Dried Frozen Sections", *J. of Histochemistry and Cytochemistry*; 14:274-287 (1966), in which frozen sections of tissue are freeze dried on emulsion coated glass slides for autoradiography. Another example is U.S. Patent No. 5,059,518, in which mammalian cells are lyophilized after being exposed to trehalose to produce a cell pellet that, when rehydrated, retains optimal physiological characteristics. Cells prepared in this fashion are disaggregated, and not cytocoherent.

Transfer of cellular and other biological components (particularly nucleic acids) on to nitrocellulose or nylon substrates has also been disclosed. See, for example, Seshi, "Cell Blotting: Techniques for Staining and Microscopical Examination of Cells Blotted on Nitrocellulose Paper", *Analytical Biochemistry*; 157:331-342 (1986); U.S. Patent No. 4,483,920 (immobilization of messenger RNA directly from cells onto nitrocellulose filter material); and U.S. Patent No. 4,455,370 (microporous nylon films used as transfer membranes for nucleic acids, proteins, bacteria and viruses).

The concept of perfusing reagents into chambers for reacting with biological materials is also disclosed in a variety of references. U.S. Patent No. 4,681,853 describes a method of perfusing reagents into a chamber holding a membrane to which nucleic acids are bound, for the purpose of treating the surfaces of the membrane. U.S. Patent No. 5,312,731 shows perfusion of cytotoxic drugs through a cell aggregate suspended in a perfusion chamber. U.S. Patent No. 4,493,815 reveals microtiter wells in a horizontal array, having perforated plates above and below the wells through which reactants are perfused. The disclosure of each of these patents is limited to exposing the surface of the specimen to reagents, or flowing reagents through disaggregated cells.

U.S. Patent No. 5,192,503 discloses a probe clip for performing in-situ assays of tissue sections, which is also discussed in McGrath et al., *Cell Vision*; 2:165-169 (1995). These references disclose a chamber with a silicon gasket which is pressed against a microscope slide to form a sealed chamber around a frozen section tissue specimen mounted on the slide. Immunocytochemical reagents are then introduced into the chamber where a reaction between the reagent and tissue specimen can occur.

In spite of years of activity in this field, significant obstacles still remain to the routine use of probes in the examination of cytological specimens. Even if a specimen is prepared by a method that maintains its cytocoherence, it must still be reacted with the probes or monoclonal

antibodies that recognize the biochemical markers associated with the cell. This is a time-consuming and expensive process. A typical colorimetric immunoassay may require 4-5 hours, including 15-30 minutes for retrieval of the specimen, 30-60 minutes for fixation, 60-90 minutes for exposure to the primary antibody, 15 minutes to wash out the primary antibody, 30-60 minutes to expose the tissue to a secondary antibody, linker or enzyme that recognizes the primary antibody to produce a colorimetric change, followed by another 15 minute wash. In situ hybridization of an oligonucleotide probe to a tissue specimen typically includes even more steps and longer incubations. These prolonged treatments reduce the efficiency of the assay and increase its cost.

It is therefore an object of this invention to provide a more efficient immunoassay of a cytocoherent specimen to detect cell markers associated with pathologic conditions.

Another object of this invention is to provide such an assay that provides reliable and more accurate results that are capable of quantitative interpretation.

SUMMARY OF THE INVENTION

The foregoing objects are achieved by the present invention, which includes a method of preparing tissue specimens for microscopic examination, by mounting a tissue specimen on a porous support that retains cytocoherence of the specimen. The tissue specimen (which may for example be a thin section, touch prep, body fluid smear, fine needle biopsy specimen, cell culture, or a biological fluid such as blood or urine) is then lyophilized in a cytocoherent form on the porous support under conditions that form microchannels in the specimen, so that a reagent can be perfused through the specimen and support. Perfusion of the reagent (such as a probe) through the porous specimen permits a rapid and effective reaction to occur between the specimen and probe. Subsequent steps, such as washing the specimen or introducing secondary antibodies, can also be performed by perfusion. Perfusion of reagents and washing solutions substantially reduces the reaction time and cost of the assay, improves the quality of the microscopic image, and enhances signal accuracy of colorimetric and other indicators used in quantitative studies.

In disclosed embodiments, the specimen is lyophilized below a eutectic temperature of the specimen. The eutectic temperature is the initial melting point of the specimen, at which water in the frozen specimen begins to liquefy (and electrical conductivity increases). Lyophilization below this temperature allows liquid to be sublimed from the solid to the gaseous phase, to form pores in the specimen through which probes and other reagents can be perfused. Lyophilization is performed under conditions that create a vertical temperature gradient, and form vertical microchannels in the specimen, to enhance the flow of reactants through the specimen. The formation of such microchannels has previously been considered an undesirable artifact, but the present invention has disregarded that teaching to arrive at the improved perfusion process.

In some disclosed embodiments, the reagent is a diagnostic antibody, which is perfused through the specimen and support at a pressure of about 10psi, and a flow rate of about 2

ml/cm²/min. Negative pressure (vacuum) as well as positive pressure can be used to perfuse the reagent through the porous specimen. The specimen may be lyophilized under conditions that form pores of about 1 μ diameter in the specimen, and a pore density of at least about 0.6×10^8 pores/cm² of tissue.

The porous support can be a composite film that includes a nitrocellulose surface layer to which the tissue specimen is adhered, and a microporous subjacent layer that has a light transmittance which is at least 60% the light transmittance of glass. The porous subjacent layer may be a microporous nylon, ceramic or cellulose material. The support can be impregnated with a detergent (such a sodium dodecyl sulfate or sodium lauryl sulfate) that improves adherence of the specimen to the support. An excipient may be added to the specimen prior to lyophilization, to normalize the eutectic temperature of the specimen at a desired process temperature, so that reproducible, uniform lyophilization will occur in specimens of varying compositions.

The reagent may be perfused through the specimen and composite film by attaching a sealed chamber to a surface of the support, and introducing the reagent in liquid form into the sealed chamber and through the specimen and film under pressure (either positive pressure or vacuum pressure). The composite film limits a rate of perfusion of the reagent, i.e. the flow rate through the composite film is lower than the flow rate through the tissue specimen. This flow limitation helps prevent uneven flow through the specimen caused by areas of reduced specimen density (such as artifactual separations), and avoids damaging the tissue with excessive flow rates.

The specimen (having a thickness of less than about 20 μ) may be lyophilized at a temperature of -70 to -30°C, for example -70 to -40°C, more specifically -40°C, to produce a pore size of about 1 μ or less. This pore size is preferred because the pores are not readily visible when microscopic images are viewed at a magnification of 400X or less (e.g. 250-400X). The specimen is also lyophilized by freezing the specimen rapidly, for example at a rate of greater than 5°C, or 20°C, 50°C or 100°C per second to promote the formation of vertical microchannels. Subsequent sublimation of ice from the frozen specimen occurs at a pressure of about .001 mm Hg, and the temperature increases during the sublimation step from -40°C (at which the specimen is held for about 1 minute) to a final temperature of +25°C over a period of 5 minutes. The final porosity of the specimen is at least 50% of the volume of the specimen.

In more specific embodiments, the method includes adhering a thin tissue specimen to a dry nitrocellulose film mounted to a porous support having an optical transmittance that is at least 60% the transmittance of glass. The porous support may be microporous nylon (such as Nytran™) having pores with a diameter of about 0.1-0.45 μ. The tissue specimen is then lyophilized under conditions that maintain its cytocoherence and form vertical microchannels in the specimen, while retaining cytocoherence of the specimen. A liquid reagent (such as a monoclonal antibody for immunophenotyping) is then perfused under pressure through the tissue specimen and porous support to react the liquid reagent with the tissue specimen. The specimen is then washed to remove

unreacted reactant, by perfusing a washing liquid under pressure through the tissue specimen and porous support. A secondary antibody may then be introduced (for example to perform an indirect assay), where the secondary antibody recognizes the primary antibody that has adhered to proteins of interest to provide a colorimetric, fluorescent or radioactive signal. This step may be followed by a second washing step to remove excess secondary antibody from the specimen.

In disclosed embodiments, the tissue specimen is lyophilized under conditions that form a lyophilized tissue specimen having pores of a diameter of less than about 1 μ and a tissue porosity of less than about 50%, and the porous composite film support is less porous than the lyophilized tissue specimen so that a rate of flow of reagent through the tissue specimen is limited by a rate of flow of the reagent through the porous support. Lyophilization of the specimen also occurs in the presence of an effective amount of a normalizing agent to normalize a eutectic temperature of the specimen, where the specimen is frozen at a rate of at least 20°C per second, and sublimation occurs for at least 1 minute at a temperature of -40 to -70°C. The liquid is perfused through the tissue specimen and porous composite film under a pressure of less than about 10 psi, and at a flow rate of less than about 2 ml/cm^2/sec, by introducing the liquid into the chamber under pressure.

In even more specific embodiments, the method of preparing the tissue specimen for microscopic examination includes providing a thin tissue specimen having a thickness of less than about 20 μ, wherein the tissue specimen is impregnated with a sufficient concentration of an excipient to normalize a eutectic temperature of the tissue specimen to a desired temperature of about -40°C. Suitable excipients include an alcohol (such as t-butyl alcohol, or high molecular weight alcohols such as polyvinyl alcohol). A composite film support is also provided, which includes a nitrocellulose surface film having a thickness of about 5 μ, a pore size of 0.01-0.8 μ and a porosity of less than about 50%, adhered to a porous nylon base, wherein the nylon base has a pore size of about 0.2 μ, a thickness of about 100 μ, and a light transmittance that is at least 60% of a light transmittance of glass. The nitrocellulose film is impregnated with a detergent that improves adherence of the tissue specimen to the nitrocellulose film, and the tissue specimen is placed on the nitrocellulose film in a dry condition so that the tissue specimen adheres with cytocoherence to the nitrocellulose film. The tissue specimen is lyophilized by placing the composite film on a freezing platen, which creates a vertical temperature gradient in the specimen, and freezing the specimen at a rate of at least 20°C. Solid ice is sublimed from the specimen, below the eutectic point, for at least one minute at a temperature of about -40°C, and at a pressure of about 1 x 10^{-3} mm Hg, to produce vertical microchannels in the tissue specimen.

A pressure chamber is then placed against the tissue specimen, to perfuse a liquid reagent containing a diagnostic antibody through the tissue specimen and composite support. The liquid reagent may be perfused at a positive or negative pressure of about 10 psi, and a flow rate of about 2 ml/cm^2/min for no more than about 1 minute, wherein the flow rate is lower through the nitrocellulose than through the tissue specimen film. A washing liquid, and/or secondary antibodies,

can subsequently be perfused through the tissue specimen and composite support. A magnified image of the tissue specimen at a magnification of about 250-400 is then examined through a microscope. In the case of a quantitative assay, such as a colorimetric reaction, the amount of the antibody or other reagent that binds to the tissue can be quantitated, for example by measuring the color intensity of the specimen.

The invention also includes a composite support for microscopic examination of a tissue specimen, wherein the composite support includes a surface film that adheres thin tissue specimens in a cytocoherent manner, and a base to which the surface film is mounted. The surface film and base are sufficiently porous to allow a reactant liquid to be perfused through the surface film and base. In disclosed embodiments, the composite support further includes a thin tissue specimen lyophilized on the surface of the film. The surface film may be a nitrocellulose film that is about 5 μ thick, has a pore size of about 0.01-0.8 μ and a porosity of less than about 50%, and the base has a pore size of about 0.2 μ, a thickness of about 100 μ, and a light transmittance that is at least 60% of a light transmittance of glass. The surface film or composite support may be impregnated with a detergent, such as sodium dodecyl sulfate or sodium lauryl sulfate, that improves adherence of the tissue specimen to the film. In particularly preferred embodiments, the base comprises a porous nylon material, such as Nytran™.

The invention also includes a system for microscopic examination of a thin tissue specimen, comprising a porous support to which the thin tissue specimen adheres in a cytocoherent fashion, and on which the thin tissue specimen can be lyophilized under conditions that form perfusion pores in the thin tissue specimen while retaining its cytocoherence. The system also includes a perfusion attachment capable of being secured to the porous support, and capable of perfusing a liquid through the thin tissue specimen and porous support under pressure without disrupting the cytocoherence of the tissue. The perfusion attachment may be a chamber with an annular seal for placement against the support.

The foregoing and other objects, features, and advantages of the invention will become more apparent from the following detailed description of a preferred embodiment which proceeds with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A is a top perspective view of a slide made in accordance with the present invention, with a porous composite film of the present invention incorporated into the slide, and with a tissue specimen on the composite film.

FIG. 1B is a cross-sectional view of a composite film on a glass slide from which the composite film may be removed for perfusion.

FIG. 1C is a cross-sectional view of a composite film on a polycarbonate slide through which a perfusion hole has been cut, so that perfusion liquid can be introduced from a perfusion tube through the composite film without removing the film from the slide.

FIG. 2 is a top view of the isolated composite film and tissue specimen, after the composite film is removed from the slide.

FIG. 3 is a cross-section scanning electron micrograph of the composite film of FIG. 2.

FIG. 4 is a photomicrograph of the microporous nylon support of the film shown in FIG. 1.

FIG. 5 is a photomicrograph of the nitrocellulose surface of the composite support, showing the pores that occupy about 50% of the surface of the nitrocellulose.

FIG. 6 is a perspective view of a perfusion apparatus for introducing immunohistochemical reagents through the tissue specimen.

FIG. 7 is an enlarged view of the perfusion chamber of the apparatus shown in FIG. 6.

FIG. 8 is an exploded perspective view of a perfusion manifold for performing cytoperfusion.

FIG. 9 is a top perspective view of a lyophilization apparatus for lyophilizing tissue specimens in accordance with the present invention.

FIG. 10 is a graph showing increased washing efficiency with the perfusion technique of the present invention, as compared to diffusional washing.

DETAILED DESCRIPTION OF SEVERAL EMBODIMENTS

The cytoperfusion technique of the present invention allows the cytocoherent transfer of proteins and nucleic acids (as well as other solutes) from porous pathological specimens (such as thin section surgical specimens from plant or animal origins) to composite supports, by lyophilizing the tissue specimen on a nitrocellulose layer of the support under conditions that promote formation of pores and vertical microchannels. The porosity of the specimen permits a probe solution to be perfused through the specimen under pressure, while retaining microanatomic relationships within the specimen. The advantage of cytoperfusion is that it greatly reduces the time needed to obtain highly sensitive results with immunoassays. Microchanneling of the specimen increases access of reactants by exposing reaction sites and creating greater surface area within the specimen for reactions to occur.

In disclosed embodiments, a specimen that is approximately 10 microns thick is freeze dried in situ on a nitrocellulose/nylon composite film, in which the nitrocellulose component has pores that are 0.1 to 0.3 microns in diameter, and the pores of the nylon component are even larger. Lyophilization conditions (temperature -40 to -70°C, below the eutectic temperature, and rate of cooling at least about 1°C per second) are selected such that pores of approximately 1 μ diameter are formed in the specimen, with an overall pore density of 30-50 percent. Uniformity of pore size can

be promoted by applying an excipient solution (such a t-butyl alcohol) that promotes formation of eutectics by normalizing the distribution of temperatures at which ice crystal formation occurs. During lyophilization, a temperature gradient is preferably created from the bottom to the top of the specimen, so that the specimen freezes from the bottom to the top. This directional freezing forms microchannels that allow perfusion of the probe reagents throughout the specimen in three dimensions. The size of the channels (directional pores) is sufficiently small not to compromise resolution of cellular microanatomy when the specimen is viewed under a light microscope at 250-400X. Perfusion of the probe reagents is performed at a pressure of 5-10 psi with a flow rate of about 2-5 ml/min/cm^2 through the tissue specimen.

One embodiment of the composite support 20 of the present invention is shown in FIGS 1A and 1B, wherein the composite support is mounted on a glass or polycarbonate slide 21. A circle of microporous nylon membrane 19 (NYTRAN from Schleicher & Schuell) having a 22 mm diameter is placed on slide 21. Polyester tape mask 22 is prepared by cutting an 18 mm diameter cut-out using a punch. The mask 22 has an adhesive undersurface that is applied to the surface of slide 21, with the cut-out in the mask 22 centered on the nylon membrane 19. A layer 26 of nitrocellulose polymer is then spun cast in a conventional fashion on to the surface of nylon membrane 19 and mask 22, so that the nitrocellulose overlaps the borders of mask 22 and occupies a circular area having a diameter that is about the same as the diameter of the nylon membrane 19.

The liquid nitrocellulose polymer from which the layer 26 is formed may, for example, be made by purchasing raw nitrocellulose materials from Hercules-Aqulon (Wilmington DE) 68.82 g RS 3/4sec, plus 68.82 g RS 18-25cps, dissolved into a mixture of 196.32 g n-butanol (purchased from Fisher Scientific, cat#·A383-4) and 7.2 g isopropanol (Fisher Scientific cat#A16P-4), and 23.10 g lauryl sulfate solution (prepared by dissolving 6.46 g of lauryl sulfate (Sigma Ultragrade cat# L-6026) in de-ionized water and stirred gently to dissolve the lauryl sulfate without foaming). The nitrocellulose polymer solution can be stored at room temperature until spin casting at 1000 rpm for 70 seconds. After spin casting, the composite film is cured at room temperature for 10 minutes, followed by baking at 60°C for one hour.

A perforation 24 is placed through layers 26, 22 and 19, and the perforation extends in the mask layer away from the composite film to form a pull-away tab 28 that can be pulled to remove the composite film from slide support 21. The composite film that is removed includes mask 22 and underlying nylon layer 19 to which mask 22 is adhered, as well as nitrocellulose layer 30 which is supported by mask 22. After placing a tissue specimen 32 on the nitrocellulose layer 30, the composite membrane and tissue specimen are placed in a perfusion device, such as that shown in FIGS. 6 and 7.

An alternative embodiment of the composite membrane is shown in FIG. 1C, which is similar to the embodiment shown in FIG. 1B, so that like parts have been given like reference numerals. However in this embodiment the slide is made of polycarbonate, and a cylindrical hole

23, about 13 mm in diameter, is provided through the slide 21. The hole 23 is filled with a porous filler (such as a porous glass plug), which provides a porous opening through which perfusion liquid can be introduced, while still providing a thermal conductance similar to the surrounding slide material 21. The nitrocellulose and Nytran layers are not separated from the slide 21 by pulling tab 28 away from slide 21 to provide an isolated composite film 31 consisting of the Nytran layer 22 and nitrocellulose layer 30. The tissue specimen 32 can be perfused in situ on the slide 21 because of the hole 23 through the slide. A perfusion tube 33 is shown in FIG. 1C, which can seat on nitrocellulose layer 30 around specimen 32 to perfuse the specimen.

Perfusion Apparatus

A simplified perfusion chamber apparatus 50 for use with the composite film of FIG. 1B is shown in FIG. 6, and an enlarged view of the perfusion chamber 52 of the apparatus is illustrated in FIG. 7. The chamber 52 is a Sweeny filter assembly having a funnel shaped base 54 with an externally threaded neck 56 that mates with an internally threaded cap 58. The composite film 31, with its adhered specimen 32, is placed on a support screen 59 that seats on the neck 56, and the gasket (O-ring) 34 seats on the composite film 31 (around specimen 32) to further seal the chamber 52 when cap 58 is mated with the threaded neck 56.

Chamber 52 is connected to a silicone rubber coupling 60, which is in turn clamped (with clamp 62) to the bottom of a 5 ml graduated pipette 64. The top of pipette 64 is in turn attached to a silicone rubber coupling 66, which is clamped with clamp 68 to a gas connector 70. Nitrogen gas tubing 72 is connected to a source of pressurized gas (not shown), and the flow of gas can be regulated by a rotatable on/off valve 74. The apparatus 50 is mounted on a ring support stand 76 with ring stand clamps 78, 80 in the conventional fashion.

In use, a reagent liquid (such as a liquid containing a monoclonal antibody) is placed in pipette 64, and perfused through composite film 30 by introducing nitrogen gas (at about 10 psi) into tubing 72. The gas pressure forces reagent liquid from the pipette 64, through composite film 30, and out of the filter assembly 52.

An alternative device for perfusing reagents through the lyophilized specimen and porous support is shown in FIG. 8, which is a modified filter manifold that can be obtained from Millipore Corporation of Bedford, MA, USA. The cytoperfusion apparatus includes a cylindrical reservoir chamber base 102 having a central positioning/locking post 104 that extends above base 102 and terminates with an externally threaded end 106. A reagent funnel 108 tapers to an outlet 110, and has an annular flange 112 around its top, peripheral to a series of outlet orifices 114. A tissue disc platform 116 which seats on funnel 108 supports a removable rigid plastic disc 117 with peripheral cut-outs in which are mounted the composite films 31. A housing 118 with a series of cylindrical reagent cups 120 seats on tissue disc platform 116 with the cups 120 aligned with the composite films 31, and a disposable rotary reagent tray 122 with a series of complementary cylindrical inserts 124

that fit within cups 120. A reagent (such as lyophilized monoclonal antibody) is adhered to the inner cylindrical surface of inserts 124. A cover 126 fits on chamber base 102 above tray 122, and is secured in place by lock nut 128. Tubing 130 communicates with the interior of apparatus 100 through cover 126.

In use, support disc 118 is placed on platform 116 with lyophilized tissue specimens on one or more of the composite films 31. The cytoperfusion manifold is assembled by placing the funnel 108 in chamber base 102, with platform 116 seated on funnel 108. Housing 118 is seated on platform 116, with reagent cups 120 aligned over composite films 30, and the cylindrical inserts 124 of the tray 122 are inserted into the complementary cups 120. Cover 126 is secured in position with lock nut 128, and pressurized liquid is introduced into apparatus 100 through tubing 130. The liquid solubilizes the lyophilized reagent in inserts 124, and perfuses the reagents through the tissue specimens on composite films 31. The perfusion liquid is collected by funnel 108, and collected in chamber 102.

The invention also includes an apparatus 130 (FIG. 9) for freezing a tissue specimen below its eutectic temperature, and providing a vertical temperature gradient that gives directionality to the pores (vertical microchanneling). Apparatus 130 includes a lyophilization chamber 132, a clear plastic cover 134, a heat sink chamber 136 below chamber 132, a series of cooling fins 133 extending in parallel rows through heat sink chamber 136 to circulate cool air, a cooling chamber 138, below heat sink chamber 136, into which liquid nitrogen is introduced, and a solid base plate 140. Six identical platens 142 are arranged in two rows within enclosure 132, and each platen has a cross-shaped recess 144 in its surface. The long arm 144a of the cross accepts an elongated slide, while the short arm 144b permits the slide to be picked up from the recess.

A liquid nitrogen inlet 150 into cooling chamber 138 communicates with a liquid nitrogen line 152, and the flow of liquid nitrogen into cooling chamber 138 is controlled by valves 154, 156. A vacuum manifold 158 communicates with lyophilization chamber 132, and leads to vacuum valve 159 and a vacuum condenser line 160, to establish a vacuum in the lyophilization chamber 132. Vacuum vent lines 162 (controlled by vent valve 164) communicate with lyophilization chamber 132 to open chamber 132 to atmospheric pressure once lyophilization has been completed.

In operation, liquid nitrogen is introduced into cooling chamber 138 through inlet 150 to precool platens 142 to -40° to -70°C. A composite film slide (like the slide 20 in FIG. 1B), with a tissue specimen 32 on the composite film, is then placed in each of the recesses 144a on the surface of platen 142. Cover 134 is secured in place to seal lyophilization chamber 132, and the interior of lyophilization chamber 132 is evacuated to a pressure of approximately 100 millitorr. After one minute of lyophilization at this temperature and pressure, the temperature of the lyophilization chamber is increased over 5 minutes to +25°C. The vacuum in lyophilization chamber 132 is then vented to atmosphere through vent 164, and the chamber 132 can be opened to remove the slide which contains the now lyophilized specimen.

Perfusion of immunocytochemical reagents through a lyophilized tissue specimen prepared in accordance with the present invention overcomes problems of poor probe binding rates (signal intensity) and non-specific probe turnover rates (noise intensity). Analyte (such as a tissue bound receptor of interest) is immobilized in the tissue by adherence to the nitrocellulose surface, hence the probe can more easily interact with the analyte in the lyophilized tissue than in solution.

The use of the specialized microporous films of the present invention, instead of non-porous glass microscope slides as specimen supports, overcomes the problem of diffusional loss of analytes by providing a relatively permeable solid phase reaction matrix to bind soluble analytes. The nitrocellulose specimen support permits vertical (capillary) transfer of soluble analytes from the specimen to the underlying film, to achieve cytocoherent transfer. Cytocoherent transfer controls diffusional loss by immobilizing analyte on a high-capacity binding matrix, which circumvents the counterproductive and antagonistic practices of "masking" analyte by over fixing the specimen and then "unmasking" the analyte by hydrolytic or other means. The binding matrix still presents analyte in solid phase, where reactions based on collision probabilities proceed more rapidly than in solution, but the regularity of the matrix and its porosity creates a reaction environment in which analyte in solid phase is uniformly more accessible to perfused probe reactants.

The present invention particularly increases precision and sensitivity of immunoassays where lyophilization occurs under conditions that promote eutectic freezing and sublimation of crystalline ice, and where pore size is controlled within certain ranges. The maximum preferred pore size corresponds with the resolving power of microscope objectives used. When the maximum magnification used is 400X, a pore size of approximately 1 μ or less is preferred. Treating tissue as a filter, and calculating permeability constants for Nucleopore standards from filtration rates and the Darcy equation, it can be estimated that a flow rate of 2-5 ml/min/cm^2 can be achieved through 10 μ thick tissue with average pore diameter of 1μ at a relatively low pressure of 5-10 psi, at an average pore density of only 30-50% (% tissue volume occupied by through- channels).

The flow rate projected from the Darcy equation for composite film supports (nitrocellulose and microporous nylon) was 0.5-2ml/min/cm^2. Perfusion rates of reagents through isolated discontinuous biological specimens (e.g. dispersed cells or coherent tissue with cracks or holes) in the specimen plane preferably exceed or are equal to the rate of flow through the film support. The composite films of the present invention achieve these differential flow rates, by casting nitrocellulose films that have smaller pores, or less porosity, than the lyophilized tissue specimen. The present examples use specimens that are at least 6 μ thick, which achieve this flow limitation. The porosity of the lyophilized tissue specimen can also be altered to affect the rate of perfusion. Porosity of the specimen can be varied by manipulating the (re)-freezing temperature during lyophilization. Generally, lower temperatures (-40 to -70°C) provide smaller pores in the lyophilized specimen than higher refreezing temperatures (such as -30°C).

EXAMPLE 1

Preparing Composite Films

To make composite films, a rectangular (20mmX20mm) sheet of nylon depth filter material ("0.2um" pore size Nytran, lot 16/9, Schleicher & Schuell, Keene, NH) was affixed to a polyester liner (M57) with a clean-release adhesive (Furon, Inc., New Haven, CT), as described in connection with FIG. 1B above. A nitrocellulose cytocoherent transfer film was then polymer cast onto the masked filter. The composite film was soaked in a detergent solution (SDS or SLS) which was dried in the material at 50°C before use. Following lyophilization, the composite films were peeled from supports and trimmed by means of a swivel-blade X-acto knife, leaving a 1.5 mm mask margin around the Film for handling and support.

EXAMPLE 2

Preparing Tissue Specimens

Breast cancers were obtained from the Anatomic Pathology laboratory, William Beaumont Hospital, Royal Oak, Michigan. Tumor fragments were quench frozen in liquid nitrogen and stored at -70°C in evacuated air-tight bags. Frozen tissue was sectioned using a Reichert-Jung cryostat at -20°C, and thaw-mounted directly onto the dry composite films, while the composite films were still affixed to microscope slides. Section thickness was 6 μ.

Freshly thawed (wet) cryostat specimens on composite films (and glass or polyester slides) were re-frozen on a pre-chilled aluminum block. Freezing continued for 5 minutes, although the specimens were visibly frozen within 1 minute. Frozen specimens (on blocks) were transferred to a thermal shelf of a Lyph-Lok 6L tray dryer (Labconco, Kansas City, Mo) with a shelf and atmosphere temperature of -40°C. Drying was at -40°C for 30 minutes at a final vacuum of 10^{-3} mm Hg (condenser temperature was -60°C).

Alternatively, lyophilization could occur in the lyophilizer 130 described in association with FIG. 9 above.

EXAMPLE 3

Immunocytochemistry Techniques

An indirect assay, employing primary antibody and either biotinylated secondary (horse anti-mouse IgG) antibody/ ABC-AP, or secondary (goat anti-mouse) antibody-AP, and BCIP/NBT-based color detection, was routinely used. Conjugated secondary antibodies and color development reagents were all obtained from Vector Laboratories (Burlingame, CA). Two primary antibodies were used: One mouse monoclonal against Her-2/neu (mAb-1, Corning Diagnostics, Alameda, CA) and a polyclonal rabbit antibody to EGFR (Ab-4; Oncogene Science, Manhasset, NY). Breast tumors were screened to provide specimens reactive with mAb-1, but not Ab-4 which then served as an antibody specificity control. Antibodies were diluted in phosphate buffered saline (pH 7.4, containing 5mM $MgCl_2$) and 0.1% Tween 20 (TPBS). Non-specific antibody binding was blocked

by pre-incubating specimens in TPBS containing either 5% normal goat serum or 1% BSA (Fraction V, Sigma, St. Louis, MO). Nuclear Fast Red (0.1%) was used to counterstain specimens (13).

To demonstrate the superiority of the perfusion technique, immunoassays were performed both by perfusion and with less efficient surface exposure techniques. The perfusion based assay was performed with a vertical volumetric column as shown in FIG. 6, which was constructed from a 1 (or 5) ml plastic pipette 64 connected to a source of dry nitrogen gas with pressure regulated in the range of 1-20 psi. Composite films 30 were inserted into the column at the base, using a Sweeny Filter Holder 52 (Gelman Sciences, Ann Arbor, MI) with a 13mm diameter filter area. The total Film area exposed was 0.81 cm^2. A mask of soft (40 durometer) silicone rubber with a 0.09 cm^2 hole was placed over the composite film to filter smaller specimen areas. Before use, fluids were pre-filtered through 0.05 μ pore size Nucleopore filters (Corning-Costar, Cambridge, MA). Flow rate through films was determined at 20°C and monitored volumetrically using a stopwatch.

Comparison assays that did not use perfusion used lyophilized specimens on composite film or glass slides, that were washed 3-4 times during the immunoassay: after primary antibody, after secondary antibody-AP (or antibody-biotin, with another wash after ABC-AP) and after substrate incubation. Each time, specimens were washed for 15 min on a rotary shaker at room temperature in staining dishes with three 250 ml changes of TPBS (5 min/change).

Specimens were stained with hematoxylin and eosin (H&E) for morphological examination. Isopropanol was substituted for ethanol in preparation of eosin. To prepare specimens for light microscopy, stained specimens were dehydrated in an isopropyl alcohol series (30-100%) and composite films were cleared to transparency by filling pores with xylene (13), which has a refractive index similar to the composite film. Specimens on composite films were permanently mounted on microscope slides in Accumount (Baxter SP, McGaw Park, Il). Specimens were viewed using a Reichert Microstar IV light microscope, employing 25X and 40X plan acromatic objectives (NA=0.5 and 0.7, respectively). The resolving power of the microscope was approximately 1 μ, based on the relationship between numerical apertures of the condenser and objective, and the wavelength of visible light (22).

The following specific examples illustrate use of the perfusion method of the present invention.

EXAMPLE 4

Perfusion Example

In accordance with the techniques described in this specification, a fresh piece of unfixed tonsil tissue, obtained as a surgical specimen, is snap frozen by submerging it in liquid nitrogen. The frozen tissue specimen is mounted on a Cryostat chuck using tissue mounting medium, and cut into a thin frozen section that is 2-10 μ thick. The thin section is thaw mounted on the nitrocellulose surface of a composite film (with a glass or polyester base), using the same technique used to apply a

frozen section to a glass microscope slide. The specimen is immediately re-frozen by placing the composite film on a platen surface of the lyophilization device (FIG. 9), in which the platens have been precooled to -40°C. The lid of the lyophilization chamber is then closed, with the lid gasket pressed tightly against the opening to form a good seal. After allowing at least 30 seconds for the specimen to re-freeze, a vacuum is established in the lyophilization chamber. The amount of vacuum may vary depending on the specimen thickness and water content, but efficient lyophilization is usually achieved in a short cycle time at 100 millitorr pressure in the chamber.

Lyophilization proceeds at -40°C for one minute. While maintaining a continuous vacuum in the chamber, the temperature of the platens is increased to +25°C over a period of 5 minutes (although the time and temperature can be varied for different specimens). At the end of this period, vacuum is maintained for 1 minute at +25°C, then the vacuum is broken. The lid of the lyophilizer is opened, and the dried samples removed. Samples are then prepared for perfusion by separating the composite film (nitrocellulose and Nytran) from the glass or polyester support by peeling or cutting, and trimming the composite film (with adhered tissue specimen) to a diameter of 13 mm.

Each trimmed sample is placed into the Sweeny filter assembly (FIG. 6) which holds a 13 mm diameter filter, such that the specimen affixed surface is positioned away from the Sweeny filter support screen. The threaded cap is used to close the assembly, and three ml of blocking solution (10% horse serum from Vector Labs, cat# S-1000) diluted in phosphate buffered saline (pH 7.5) containing 0.3% Tween-20 (TPBS) which has been pre-filtered through a 0.2 μ cellulose acetate filter is placed into a 5 ml graduated pipette. The pipette is supported in a vertical orientation by a ring stand to which the pipette is clamped.

The top of the pipette is attached to a pressurized tank of nitrogen gas through a connector with an on/off valve and an adjustable pressure gauge set to 10 psi. The lower tip of the pipette is fitted with a piece of flexible tubing with a 1/8 inch inner diameter, and the threaded portion of the Sweeny filter assembly is connected to the pipette by inserting the threaded exterior portion of the cap into the silicone tubing. Reagent flow through the specimen is started by opening the pressure valve at the top of the reagent pipette, and 2 ml of blocking solution is delivered through the specimen at 10 psi. A typical specimen will allow reagent to perfuse through the specimen at a rate of 2 ml/min at 10 psi. The surface of the specimen is constantly bathed in reagent solution and not allowed to dry.

After delivery of 2 ml of blocking solution, reagent flow is stopped by turning off nitrogen gas flow. The pipette is temporarily disconnected from the Sweeny filter and filled with 0.5 ml of diluted primary antibody reagent solution, such as anti-PCNA mouse antibody (Vector Labs, Burlingame, CA., Cat#NCL-PCNA). Dilution of primary antibody is made in TPBS containing 1.0% normal horse serum (Vector Labs, Cat# S-2000). After re-connecting the Sweeny filter to the pipette, reagent flow is started again. After delivery of the 0.5 ml of reagent into the

Sweeny filter, gas pressure is interrupted and the specimen is allowed to soak in primary antibody reagent for 5 minutes. After 5 minutes of primary incubation, the pipette is refilled with 2 ml of wash buffer (TPBS without serum), and delivered through the specimen as above.

Secondary antibody is then delivered in the same fashion as above. In this Example, the secondary antibody is Biotin labeled anti-mouse antibody (from Vector Labs # BA2000) diluted 1:2000 in pre-filtered TPBS containing 1% horse serum. The serum is then washed as already described, this time to remove the secondary antibody. Fluorescent cetection is performed using fluorescein anti-biotin reagent (Vector Labs # SP3040) prepared in TPBS and using the same 5 minute incubation described above. The specimen is then perfusion washed with de-ionized water to stop the incubation. The specimen is removed from the Sweeny filter assembly and mounted with aqueous mounting medium suitable for use in fluorescence microscopy (Vector Labs VectaShield reagent # H-100), a cover slip is placed over the specimen, and it is viewed microscopically, using the same wavelength and filters conventionally used for fluorescent imaging. 0.5 ml of alkaline phosphatase labeled anti-mouse antibody (Vector Labs, Cat#AP-2000) diluted 1:2000 in pre-filtered TPBS containing 1.0% horse serum.

Alternatively, colorimetric detection may be preformed using alkaline phosphatase substrate incubation (with Vector Labs Alkaline Phosphatase Substrate Kit, Cat#SK5200 prepared in 0.1M TRIS solution, at pH 9.5, as instructed by the manufacturer), and using the same 5 minute incubation as described for the primary antibody. Washing solution (deionized water) may be perfused through the specimen in the Sweeny filter to stop color development.

The specimen, after being removed from the Sweeny filter assembly, may be rendered optically clear by dipping the composite film in xylene several times. The sample may be prepared for microscopic viewing by placing a drop of mounting medium such as Accumount (Baxter Scientific) on to a glass microscope slide. The composite filter is placed on top of the drop of medium, with the tissue specimen face up, and 2-3 drops more of medium are applied on the specimen surface and overlaid with a glass cover slip. The finished specimen may be viewed microscopically for the presence of brown color stain in the nucleus, which indicates the presence of PCNA protein.

EXAMPLE 5

Alternative Perfusion Technique

A block of formalin fixed paraffin embedded with human tonsil is mounted on a microtome chuck, and 4 μ thick sections of tissue are made. The tissue specimens are floated on the surface of a water bath, and each thin section is placed on a separate composite film. The tissue specimen is affixed to the nitrocellulose surface of the composite film by microwave heating for 1 minute, or baking at 60°C for one hour. The tissue is de-paraffinized by incubation in xylene, and descending concentrations of isopropyl alcohols, using the following sequence: xylene, xylene, xylene, followed by sequential isopropyl alcohol treatments for 3 minutes at each of the following

concentrations: 100%, 100%, 100%, 95%, 90%, 80%, 70%, and then water. The distribution of water in the specimen is normalized by soaking the specimen for one minute in a 75% tertiary butyl alcohol (TBA) excipient. A disclosed embodiment of the excipient is 75% TBA, 5% sodium lauryl sulfate (SLS), 1 % polyvinyl alcohol (PVA), 1% polyethylene glycol (PEG), 10% gelatin, and 1% Tween-20.

The composite films (with the attached tissue specimens) are removed from the normalizing solution and excess solution is drained on to paper towels. The specimen is frozen by placing the composite film on the platen of the lyophilization device (FIG. 9), in which the platen surface temperature has been pre-cooled to -45°C (the condenser surface is cooled to below -60°C). Composite films with attached tissue specimens are inserted on different platens until all the available freezing platens are occupied. The lid is closed, and a gasket pressed tightly against the opening to form a good seal when vacuum is applied. After allowing at least 1 minute for the last specimen to freeze, a vacuum of 100 millitorr is applied in the lyophilization chamber. The tonsil specimens are lyophilized at -45°C for 3 minutes. Then while maintaining a continuous vacuum of 100 millitorr in the lyophilization chamber, the temperature of the sample platens is increased to +25°C over a period of 10 minutes. After reaching this temperature the vacuum is maintained for 1 minute before venting the chamber to atmosphere, opening the lid, and removing the sample.

Samples are prepared for perfusion of reagents by peeling or cutting the composite film (nitrocellulose and Nytran) from the support, and trimming to 13 mm. The composite films are placed in the Sweeny filter and perfused as described in Example 4 with the blocking solution, primary antibody, washing solution, secondary antibody, washing solution, and colorimetric reagent. Dehydration and preparation for microscopic viewing are performed as in Example 4.

Fluorescent antibody detection can be performed by delivering antibody as described above, but where the antibody is Biotin Labeled Anti-mouse antibody (Vector Labs #BA2000) diluted 1:200 in pre-filtered TPBS containing 1% horse serum. The specimen is then washed as already described, this time to remove the secondary antibody. Fluorescent detection is performed using fluorescein-anti-biotin reagent (Vector Labs # SP3040) prepared in TPBS and using the same 5 minute incubation as described. Specimen is then perfusion washed with de-ionized water to stop incubation. The specimen is removed from the Sweeny filter assembly and mounted with aqueous mounting medium suitable for use in fluorescence microscopy (Vector Labs, VectaShield reagent # H-1000), cover-slipped and viewed microscopically at the same length and filters conventionally used for fluorescent imaging.

EXAMPLE 6

Properties of Composite Films

FIGS. 2-5 illustrate key physical properties of the composite film 31, which are also summarized in Table 1. FIG. 2 shows a composite film with a lyophilized cryostat breast cancer

specimen shown after a Her-2/neu immunoassay. FIG. 3 is a cross-section scanning electron micrograph of the composite film with the tissue specimen affixed to the nitrocellulose surface. Nitrocellulose(NC)/Nytran(NY) composites were 185 μ thick, with the exposed nitrocellulose surface layer comprising approximately 5 μ. The nitrocellulose polymer penetrated the superficial nylon latticework (FIG. 4) to a depth of about 5 μ to form an intimate connection between the two materials, with no air pockets or other discontinuities visible at 3000-10,000 X. The nitrocellulose surface of the composite formed a smooth contact plane for biological specimens, as shown by the intimate contact between tissue specimen (T) and film in FIG. 3.

FIGS. 4 and 5 contrast the surface of a Nytran™ filter and a composite film having a nitrocellulose surface. The Nytran surface (FIG. 4) comprises an "open meshwork" structure without discrete pores, while the working nitrocellulose surface (FIG. 5) of the composite film is planar with discrete round pores comprising approximately 50% of surface area (3.6×10^8 pores/cm^2). The range in pore size on the surface of the nitrocellulose films was 0.01-0.8 μ. Approximately 91% of pores were 0.2-0.4 μ in diameter (Table 1); 10% were 0.4-0.8 μ, and 10% were <0.2 μ.

Table 1

Pore Structure and Permeability of Composite Films.

Film	Surface Pore Diameter[b] (μ)	Surface Porosity[b] (pores/cm^2)	Thickness (μ)	Flow Rate[c] (ml/min/cm^2)	Permeability[d] K[d](x10^{-10})
Film composite	0.2 - 0.4	$3.5 \times 10^8 \pm 0.1 \times 10^8$	185 ± 3	2.03 ± 0.21	10 (0.27)[e]
Nucleopore standard	0.2	$3 \times 10^8 \pm 0.03 \times 10^8$	10 ± 0.1	17 ± 0.9	4
Nucleopore standard	0.4	$1 \times 10^8 \pm 0.01 \times 10^8$	10 ± 0.1	62 ± 3.3	15
Nytran[a] (0.2 μ)	"open weave"	Nf.	180 ± 3	24 ± 0.6	105

[a] The surface porosity of Nytran (0.2 μ pore size) is not relevant (NR) because of the "open weave" structure of the surface.

[b] Surface porosity and pore diameter were measured directly from scanning electron micrographs.

[c] Flow rate of pre-filtered distilled deionized water was measured at four 0.09 cm^2 sites in five Films from separate lots.

[d] A permeability constant (K in cm^2) was determined from the Darcy equation $K = \frac{q \times u \times L}{\Delta P \times A}$ where q = flow rate (cm^3 / sec), u = viscosity, taken as 1.0 (dyne sec / cm^2), L = thickness (cm), ΔP = pressure (dynes / cm^2), A = surface area (cm^2).

[e] Permeability calculated as a single uniform resistance (10cm^2) and as $R_{total} - R_{Nytran}$ (0.27 cm^2)

Table 1 also shows the permeability of laminate composite films to water under positive pressure. At 10 psi, the rate of water flow through laminate films was approximately 2 ml/min/cm^2,

an average of 19 times slower than through Nucleopore films of the same surface porosity, with etched pores of the same diameter (0.2 μ-0.4 μ). The rate of water flow through composite films was approximately 10 times slower than through the nylon support filter alone (Table 1), suggesting that the nitrocellulose thin film component of the composite determined permeability of the composite.

Permeability of composite films was within the range established for Nucleopore standards of the same pore size and porosity (Table 1). However, since the 10 μ thick nitrocellulose component of the 185 μ thick composite film actually determined flow rate through the entire composite, we could calculate from the Darcy equation, which relates thickness to flow rate and permeability, that the cast nitrocellulose film was actually 15 to 55 times less permeable (more tortuous) than the etched Nucleopore films of the same thickness with regular cylindrical pores.

EXAMPLE 7

Formation Of Pores In Specimen By Lyophilization

Lyophilization created pores in cryostat tissue specimens which were readily visible at the tissue surface in scanning electron micrographs. Table 2 shows representative effects of lyophilization on the size and density of pores at the surface of tissue sections. Data obtained from studies of three breast tumor specimens are summarized in Table 2. Both freezing and vacuum drying were necessary for pore formation, suggesting that pores were formed by sublimation of crystalline ice. Requirement for ice crystals in pore formation was also demonstrated by the absence of pores in vitrified (in 30% sucrose) specimens (data not shown).

The porosity of tissue was sensitive to the temperature at which thaw-mounted cryostat specimens were re-frozen for lyophilization. Mean pore diameter increased about 4 times, from an average of 0.392 μ to an average of 1.6 μ, as freezing temperature was raised from -70°C to -30°C, suggesting that pore size was a function of the re-freezing rate of ice in the specimen which determined the size of ice crystals.

The percent of tissue surface which comprised pores (porosity) also increased with pore size and re-freezing temperature, from a minimum of 30% at -70°C to a maximum of 65% at -30°C. The distribution of pores was relatively homogeneous within specimens re-frozen at -30°C and -40°C, whereas at -70°C, there were areas of specimen with no visible pores. Specimens re-frozen at -20°C were relatively poreless, with clusters of a few large vacuole-like pores, and appeared more "congealed," suggesting that the tissue may have collapsed due to formation of excessively large pores and involvement of too much of the tissue matrix.

The variation in pore size and surface porosity was greatest at a refreezing temperature of -30°C (Table 2), which approximates the eutectic freezing temperature of tissue. Surface involvement was more homogeneous in specimens refrozen at -40°C, where pore density at the porous tissue surface was determined to be 0.6×10^5 (Table 2), approximately 5-fold lower than the surface porosity of composite films.

Table 2

Effect of re-freezing temperature of lyophilization on tissue porosity and Her-2 signal precision.

Refreezing Temperature (°C)	Pore Diameter (μ)	Tissue Porosity % (pores / cm²)	Her-2 Signal Precision
NF[a] (vacuum dry)	-	0	+ +
-70	0.392 ± 0.163	30 - 40	+ + + +
-40	0.980 ± 0.308	43 - 50 ($0.6 \times 10^{4} \pm 0.1 \times 10^{4}$)	+ + + +
- 40 (air dry)	-	0	+
- 30	1.6 ± 0.899	55 - 65	+ + +
- 20	0.221 ± 0.131	5 - 10	+ +

[a] NF = tissue not refrozen

[b] Tissue pore diameter and porosity were measured from scanning electron micrographs. Mean and S.D. in pore diameter are given for three separate tumors (2 specimens/tumor). Porosity is expressed as a range in % of specimen surface involved and where statistically meaningful ($p < 0.05$), pore density for the same specimens.

[c] Precision of color signal at carcinoma cell membranes is expressed in a scale of 1+ to 4+, where 1+ is 10% of cells and 4+ is 90% of cells with coherent membrane signal (2+ = > 10% < 50% , 3+ = > 50% < 90%). Determinations are from five, 250X microscope fields in paired sections from 3 breast cancers.

EXAMPLE 8

Effect Of Pores on Cell Morphology and Her-2/neu signal precision at cell membranes

Pores formed by lyophilization at freezing temperatures of -70°C and -40°C were not readily visible in specimens observed at 400 X after chemical fixation and staining. Furthermore, neither the presence of pores in the range of 1 μ (diameter) or less, nor the process of forming those pores, created anatomical artifacts that impaired cytology. Major cell types could readily be differentiated, including glandular epithelial, myoepithelial, endothelial and lymphoid in lyophilized breast cancer tissue re-frozen at -70°C and -40°C. Major breast cancer growth patterns could also be differentiated, including papillary, cribiform and comedo. Mitotic figures were readily discernable in lyophilized specimens re-frozen at -70°C and -40°C. At higher freezing temperatures (-30°C and -20°C), vacuoles were readily visible in fixed and stained specimens examined at 250 X. Cell matrices and mitotic figures were indistinct at these freezing temperatures. Spaces between cells were observed in specimens whether frozen or not. These spaces were artifacts related to the stretching of film during dehydration and not to the formation of pores.

The formation of pores in the specimen of 1 μ diameter or less did not reduce precision of Her-2 signal at cell membranes (Table 2). Signal was actually more precise in porous lyophilized specimens which had been re-frozen at -70°C and -40°C than in non-porous specimens which had

been vacuum dried rather than lyophilized. Signal precision was reduced in lyophilized specimens which had been frozen at -30°C and -20°C, heavily vacuolated specimens which were morphologically indistinct. The virtual loss of Her-2 signal at carcinoma cell membranes in specimens which had been frozen at -40°C and air dried (Table 2), was presumably the result of antigen diffusion within the porous film matrix because specimens were not fixed before Her-2 assay.

Flow rate through tissue was directly related to it's porosity; air-dried tissue with no pores was essentially impermeable to TPBS at 10 psi, and flow-rate through lyophilized tissue pre-frozen at -70°C was 0.6 ml/min/cm^2.

Assay sensitivity was not affected by filtration, which indicates that the velocity of perfusion-based washing did not create sufficient shear force to displace specifically bound antibody. Likewise, filtration at 10 psi pressure did not affect the precision of Her-2 signal at cell membranes or cell morphology in tissue that was also examined at 400 X.

EXAMPLE 9

Perfusion Based Washing More Efficient

The time required to remove non-specifically bound primary antibody from tissue to background levels (washing efficiency) was reduced by a factor of 15 using perfusion washing of porous lyophilized specimens compared to conventional diffusion-based washing of non-porous specimens (FIG. 10). One minute perfusing lyophilized tissue with TPBS at 10 psi was required to reduce non-specific antibody content to background levels. By one minute of diffusion-based washing, backgrounds were reduced erratically in specimens by an average of only 40%. At one minute of perfusion-based washing, only 1.9 ml TPBS was used, in contrast to 250 ml used for diffusion washing. Finding that non-specific (EGFR) antibody content of lyophilized tissue was reduced to a consistent background at multiple sites in perfused specimens suggested that the entire porous matrix of the specimen was permeable to TPBS under the conditions of filtration shown.

The filtration loading capacity of specimens on composite films was established using purified BSA pre-filtered through 0.2 μ pore size Nucleopore filters. At 10 psi, the flow rate through a specimen on composite film was reduced by 20% after passage of 15 ml BSA (10 mg/ml in TPBS). Flow rate was reduced by 40% after filtering 22 ml. The highly efficient washing of non-specific antibody from tissue was likely related to the ability of the perfusion process to perfuse in excess of 10^4 tissue void volumes of buffer in less than one minute. Performing all washes for a single Her-2 assay in perfusion format would reduce assay time by about 45 minutes and save nearly 750 ml of buffer.

Magnifications for marker analysis in situ are typically 250-400 X. Spatial resolution at these magnifications is in the 1 μ range, which was also the range in pore diameter that allowed the most efficient perfusion of immunocytochemical reagents through the specimen. Thus at 250 X magnification, pore sizes less than 1 μ were not likely visible, and did not change contrast artifacts associated with resolution of cellular structures in stained specimens. Ice crystal and resultant pore

size is inversely related to the rate at which ice re-freezes, hence lower temperatures provide larger diameter pores while higher temperatures provide smaller pores. Surface pore diameter and internal pore diameter were virtually identical at a given freezing temperature. At a re-freezing temperature of -40°C, pore diameter was nearly 1 μ, and variation in pore size was only 31% in multiple areas of three different breast tumors. The internal porosity of these specimens was vertically directional, giving the appearance of vertical "microchannels".

Although the above examples were performed with a coherent piece of solid tissue, which provided a stable fabric to lyophilize, sufficiently large pores can be introduced into dispersed cells for perfusion, while still maintaining morphological integrity. These large pores allow the specimen to be made sufficiently permeable so that the composite film and not the specimen is rate limiting for reagent flowthrough.

The use of detergent-impregnated Film can contribute to the stability of microchannelled tissue. On glass microscope slides, crystalline (microchannelled) specimens have increased fragility. The detergent (such as SDS or SLS) can "weld" the specimen to a nitrocellulose film surface with an exceptionally high surface area for attachment.

The perfusion technology of the present invention can substantially increase the efficiency of cytochemical assays. A typical colorimetric immunoassay comprises 8 basic steps involving 4-5 hours (retrieval 15-30 minutes; blocking, 30-60 minutes; primary antibody, 60-90 minutes; wash, 15 minutes; secondary antibody/linker/enzyme, 30-60 minutes; wash 15 minutes; substrate, 30 minutes; wash, 15 minutes). In situ hybridization typically comprises even more steps and longer incubations. However, by percolating reactants into porous tissue under pressure, we anticipate blocking and probe incubations will be completed within one minute. This savings in time will be matched by savings in reagent cost, because much smaller volumes of reagents need to be used in the more efficient perfusion assays. Perfusion assays can be performed with microliter volumes of expensive reagents, instead of the liters of reagents that are used in the prior art.

EXAMPLE 10

Fabrication of Composite Films

The composite films 31 (FIG. 1) are manufactured by a process of placing a clean-release adhesive liner over a depth (or other) filter to expose a circular filter area, using the liner to mask the filter and adhere it to a glass microscope slide. Affixing the composite film to a microscope slide facilitates tissue mounting and heat transfer during freezing and drying, and the film can be easily removed from the slide for filtering. Composite films are polymer cast onto the exposed filter area. After a quench process to form pores, a detergent was added and dried on the Film.

The nitrocellulose films can be cast on a variety of microporous support materials, which are shown in Table 3. Nytran was preferred because it has a 0.2 μ pore size (pore size in these depth filters is a measure of particle exclusion limit rather than surface or internal pore size), an exceptionally smooth surface, tough pliability, absence of a fibrous core, and chemical properties that promoted nitrocellulose bonding without dissolving in the acetone solvent or pore formers. The

0.2 μ pore size was selected over 0.45 μ, because that smaller pore size provides an optimally smooth surface for polymer casting, while still preserving adequate flow characteristics.

Table 3

SUPPORT						COMPOSITE			
Material	Mfg.	Pore Size (um)	Thickness (um)	Flexible Strength	Chemical "Melt"[4]	Linear Distort.[5]	Light Transm[6] %	Focal Uniform[7] %	Cytol Resol[8] %
cellulose nitrate	S & S[1]	0.45[2]	160	+ +	+	19.7	99.7	NT	NT
cellulose acetate	S & S	0.45	160	+ +	+	4.5	87	NT	NT
Durapore (PVDF)	Millipore	0.2	180	+ + + +	-	0	8	NT	NT
Supor (polysulfone)	Gelman	0.2	120	+ + +	-	0	0.4	NT	NT
Nuclepore (PC)	Corning/ Costar	0.2	10	+ + +	-	buckled	27.0	NT	NT
Nytran	S & S	0.2	180	+ + + +	-	10	71	100	100
Max Strength Nytran	S & S	0.2	180	+ + + +	-	10	68	100	100
Glass Microfibre	Whatman	-	250	+ + +	-	0	55	44	NT

[1] Schleicher & Schuell

[2] Minimum particle size excluded as per mfg. Actual pore size is much (at least 10X) greater, There are no defined pores on the surface of 0.2 or 0.45 filters.

[3] a relative measure of ease with which composites could be broken or pulled apart by hand, (+ + + +) = very difficult, (-) = support fell apart during routine handling.

[4] micromelting of support during Oncyte Film casting process prevented uniform gas bubble formation under pressure

[5] Measured at 50X using an eyepiece micrometer, before and after soaking laminates in 100% isopropanol (IPA), % = % linear swelling of composite in IPA

[6] % light transmitted through xylene-soaked composite at 530nm (relative to glass slide = 100%)

[7] % of 30 microscope fields (250X) selected at random in which all nuclei of cells in a 2um thin section of hematoxylin-stained tissue affixed to the surface of laminates remained in the same focal plane.

[8] Ability to resolve secretory ep. and myoep. cells and mitotic figures in 6um sections of hematoxylin-stained tissue affixed to the Oncyte surface of composites (% concordance in paired sections between test surface and glass slides).

The Nytran that is chosen preferably has light transmittance of at least 60% of glass (i.e. the percent of visible and UV light that is transmitted though the Nytran should be at least 60% of the same light that would be transmitted through glass of the same thickness). Maximum Strength Nytran (also without internal fiber support), can be equivalent to the Nytran in all properties shown in Table 3 (including light transmittance). Neither product autofluoresced in ultraviolet light. However, the refractive indices of nylon and nitrocellulose should ideally be identical. Another potential support material is nylon stretched 10% of its length in isopropanol, but this support allows the nitrocellulose film to stretch, which can cause break artifacts in the specimen matrix.

Other potential porous support materials include porous glass coverslips (0.5 pore size from Corning/Kamperman); etched ceramic filters (0.2 μ pore size from Whatman, Anopore); and regenerated cellulose (0.2 μ pore size from Schleicher & Schuell). The glass, ceramic and

regenerated cellulose materials have suitable visible/UV light transmittance and stretch-resistance. When porous glass or ceramic is used as the support, a silane can be used to covalently bond the nitrocellulose to the glass.

A suitable polished glass material which is 200 μ thick (the approximate thickness of a #1 cover slip), with pores approximately 0.5 μ in diameter, is available from Corning (Vycor Brand). Thin (60 μ) ceramic Anopore filters from Whatman are also suitable. The porous support material preferably has at least 60% light transmittance, no autofluorescence in ultraviolet light, 100% focal plane uniformity, less than 1% linear distortion in 100% isopropanol, and 100% cytologic resolution.

EXAMPLE 11

Lyophilization Parameters

Lyophilization of the tissue specimen can be performed under a variety of conditions, with the goal of providing a porous specimen with vertical microchannels through which a liquid can be perfused. This goal can be achieved by a device (such as that shown in FIG. 9) which freezes the specimens a rate greater than 5°C/sec (for example 20°C/sec to 100°C/sec or more), under well regulated vacuum and temperature control. A 1:1 ratio between drying surface area and condenser surface area is adequate for optimum condenser efficiency.

Precise control of temperature maximizes vapor pressure and controls ice recrystalization rates. Similarly, rapid chamber evacuation provides optimum crystal size and directionality during primary drying. The drying cycle is initiated when a crystalline state is achieved by cooling to -70°C (with liquid nitrogen) and in a low pressure environment, providing a vapor pressure differential at the specimen. The vapor pressure differential and vapor flow conditions in the instrument will be sufficient to sublime specimen ice (70-500 μL) in approximately one minute at -40°C.

EXAMPLE 12

Use of Detergent and Excipient

In disclosed embodiments, composite films were soaked in an excipient containing 75% tertiary butyl alcohol (TBA), 5% sodium lauryl sulfate (SLS), 1 % polyvinyl alcohol (PVA), 1% polyethylene glycol (PEG), 10% gelatin, and 1% Tween-20. The composite films may also be soaked in a detergent such as SLS or SDS, which was then dried on the surfaces of the composite films. The detergent plasticized the film, welded the specimen to film, extracted weakly (non-covalently) bound membrane proteins, and enhanced vertical water/solute transfer to film through capillary action. The detergent also apparently improved the uniformity of solute water distribution (ice nucleation sites) within specimen. The detergent is also believed to normalize (by lowering) the eutectic freezing temperature, and may also chemically help to form pores in specimen.

The addition of an excipient (such as 75 % t-butyl-alcohol) to the tissue specimen prior to lyophilization also helps improve uniform distribution of water in the specimen, enhances nucleation of ice, normalizes the eutectic temperature and refreezing rate of ice in specimens that are otherwise intrinsically heterogeneous with respect to these properties, and helps preserve the structural stability

of porous specimens and labile analytes in the dried state. The excipient is believed to normalize the eutectic temperature (to about -38°C) by imposing its own water content and solvent distribution on the specimen, which is greater than the inherent water concentration of the specimen.

EXAMPLE 13

Hybridization

The hybridization assay we employ is essentially that of Lawrence and Singer, Nucleic Acids Res. 13:1777-1799, 1985, using a full length cDNA and hybridization at 42°C in 50% formamide, except that digoxygenin-labeled cDNA is employed and hybridization is detected using FITC-labeled anti-digoxygenin antibody. The composite film assay format also differs from the glass slide format in that the protease digestion step is omitted as unnecessary for lyophilized specimens. Data are obtained using a Bio-Rad Image Analyzer, and expressed as % reactive cells (the target is diffusely cytoplasmic at 250-400 X), with variation determined using a Factorial Anova with nested components.

EXAMPLE 14

Quantifying Immunocytochemical Results

The intensity of a colorimetric signal can be quantitated, in accordance with known methods. A photomicrograph of the specimen can be obtained, and the images digitized. A pixel intensity scan is taken along a computer generated line between markers on the digital image, and the intensity of pixels determined along that line. The maximum pixel intensity is an indication of the concentration of the probe (such as a monoclonal antibody) that has hybridized to the tissue. The intensity of this signal can in turn be correlated with the concentration of the protein of interest in the tissue.

Pathology specimens that are suitable for immunohistochemical analysis include cells that express labile proteins and mRNA markers (for example Her-2, PgR, EMA, A blood group, and b-actin), from any type of animal or plant tissue. The specimen thickness may vary widely, for example from 1 μ to 100 μ (although specimens may be outside this and other ranges given in the specification).

The thickness of the composite film (Nytran and nitrocellulose) can also have a variety of range of thicknesses, as long as the thickness does not interfere with imaging and stain backgrounds. A thickness of less than 200 μ (for example 185 μ or less) is an example of a suitable thickness.

Perfusion flow rates can also vary widely, but are optimally as fast as possible without compromising the specimen structure, for example 0.5 ml/min/cm^2 to 5 ml/min/cm^2. Perfusion pressure is optimally less than 30 psi.

A range of exemplary lyophilization temperatures is -20°C to -70°C, at a lyophilization pressure of 200 millitorr to 70 millitorr.

Having illustrated and described the principles of the invention in several different embodiments, it should be apparent to those skilled in the art that the invention can be modified in arrangement and detail without departing from such principles. We claim all modifications coming within the spirit and scope of the following claims.

We claim:

1. A method of preparing tissue specimens for microscopic examination, comprising:

mounting a tissue specimen on a porous support that retains cytocoherence of the specimen;

lyophilizing the specimen on the porous support under conditions that form microchannels in the specimen; and

perfusing a reagent through the specimen and support;

2. The method of claim 1, wherein the specimen is lyophilized below a eutectic temperature of the specimen.

3. The method of claim 1, further comprising examining the specimen for the presence of a reaction between the specimen and the reagent that indicates the presence of a pathological condition associated with the specimen.

4. The method of claim 1, wherein the reagent is a diagnostic antibody.

5. The method of claim 1, wherein the reagent is perfused through the specimen and support at a pressure of about 10 psi, and a flow rate of about 2 ml/min/cm2.

6. The method of claim 1, wherein the specimen is lyophilized on the porous support under conditions that promote the formation of vertical microchannels in the specimen.

7. The method of claim 1, wherein the specimen is lyophilized under conditions that form pores of about 1 μ diameter in the specimen.

8. The method of claim 1, wherein the specimen is lyophilized under conditions that provide a pore density of about 0.6×10^8 pores/cm^2.

9. The method of claim 1, wherein the support comprises a composite film that includes a nitrocellulose layer in contact with the tissue specimen, and a porous subjacent layer that is has a light transmittance that is at least 60% the light transmittance of glass.

10. The method of claim 9, wherein the porous subjacent layer comprises a material selected from the group consisting of microporous nylon, ceramic, and cellulose.

11. The method of claim 1, further comprising impregnating the support with a detergent that improves adherence of the specimen to the support.

12. The method of claim 1, further comprising adding an excipient to the specimen prior to lyophilization, wherein the excipient normalizes the eutectic temperature of the specimen.

13. The method of claim 1, wherein perfusing a reagent through the specimen and support comprises attaching a sealed chamber to a surface of the support, and introducing the reagent in liquid form into the sealed chamber and through the support under pressure.

14. The method of claim 1, wherein the support limits a rate of perfusion of the reagent through the support and specimen.

15. The method of claim 1, wherein the specimen is lyophilized at a temperature of -70 to -30°C.

16. The method of claim 15, wherein the specimen is lyophilized by freezing the specimen at a rate of more than 5°C per second.

17. The method of claim 16, wherein the specimen is a thin section tissue specimen less than about 20 μ thick prior to lyophilization.

18. A method of preparing a tissue specimen for microscopic examination, comprising:

adhering a tissue specimen to a dry nitrocellulose film mounted on a porous support having an optical transmittance at least 60% of glass, the porous support having pores with a diameter of about 0.1-0.45 μ; and

lyophilizing the tissue specimen under conditions that form vertical microchannels in the specimen, while retaining cytocoherence of the specimen.

19. The method of claim 18, further comprising perfusing a liquid reagent under pressure through the tissue specimen and porous support to react the liquid reagent with the tissue specimen.

20. The method of claim 19, further comprising washing the specimen to remove unreacted reactant, by perfusing a washing liquid under pressure through the tissue specimen and porous support.

21. The method of claim 18, wherein the tissue specimen is lyophilized under conditions that form a lyophilized tissue specimen having pores of a diameter of less than about 1 μ and a tissue porosity of less than about 50%, and the nitrocellulose film is less porous than the lyophilized tissue specimen so that a rate of flow of reagent through the tissue specimen is limited by a rate of flow of the reagent through the nitrocellulose.

22. The method of claim 21, further comprising adding a normalizing agent to the tissue specimen prior to lyophilizing the tissue specimen, to normalize a eutectic temperature of the specimen.

23. The method of claim 19, wherein the liquid is perfused through the tissue specimen and porous support under a pressure of less than about 10 psi, and at a flow rate of less than about 2 $ml/cm^2/min$.

24. The method of claim 19, wherein the liquid is perfused through the tissue specimen and support by attaching a chamber to the surface of the tissue specimen, and introducing the liquid into the chamber under pressure.

25. The method of claim 18, wherein the tissue specimen is lyophilized by freezing at a rate of at least 20°C per second, and sublimation for at least 1 minute at a temperature of -40 to -70°C.

26. A method of preparing a tissue specimen for microscopic examination, comprising:

providing a thin tissue specimen having a thickness of less than about 20 μ, wherein the tissue specimen is impregnated with a sufficient concentration of an alcohol to normalize a eutectic temperature of the tissue specimen to a desired temperature of about -40°C;

providing a composite porous support comprising a nitrocellulose film having a thickness of about 5 μ, a pore size of 0.01 to 0.8 μ, and a porosity of less than about 50%, adhered to a porous

nylon base, wherein the nylon base has a pore size of about 0.2 μ, a thickness of about 100 μ, and a light transmittance that is at least 60% of a light transmittance of glass;

impregnating the nitrocellulose film with a detergent that improves adherence of the tissue specimen to the nitrocellulose film;

placing the tissue specimen on the nitrocellulose film in a dry condition so that the tissue specimen adheres with cytocoherence to the nitrocellulose film;

lyophilizing the tissue specimen on the support by freezing the specimen at a rate of at least 20°C per second, followed by sublimation of solid ice for at least one minute at a temperature of about -40°C and at a final pressure of about 1 x 10^{-3} mm Hg to produce vertical microchannels in the tissue specimen;

placing a pressurized chamber over the tissue specimen, perfusing through the tissue specimen and composite porous support a liquid reagent containing a diagnostic antibody, wherein the liquid reagent is perfused at a pressure of about 10 psi and a flow rate of about 2 ml/cm^2/min for no more than about 1 minute, wherein the flow rate is lower through the nitrocellulose film than through the tissue specimen, then perfusing a washing liquid through the tissue specimen and composite support for no more than about 1 minute; and

examining a magnified image of the tissue specimen at a magnification of about 250-400.

27. The method of claim 26, further comprising quantitating a reaction between the reagent and the tissue specimen.

28. A composite support for microscopic examination of a tissue specimen, comprising:

a surface film that adheres thin tissue specimens in a cytocoherent manner; and

a base to which the surface film is mounted, wherein the surface film and base are sufficiently porous to allow a reactant liquid to be perfused through the surface film and base.

29. The composite support of claim 28, further comprising a thin tissue specimen lyophilized on the surface of the film.

30. The composite film of claim 28, wherein the surface film comprises a nitrocellulose film.

31. The composite film of claim 28, wherein the film is about 5 μ thick, has a pore size of about 0.01-0.8 μ and a porosity of less than about 50%, and the base has a pore size of about 0.2 μ, a thickness of about 100 μ, and a light transmittance that is at least 60% of a light transmittance of glass.

32. The composite film of claim 28, wherein the composite support is impregnated with a detergent that improves adherence of the tissue specimen to the film.

33. The composite film of claim 32, wherein the detergent is sodium dodecyl sulfate or sodium lauryl sulfate.

34. The composite film of claim 31, wherein the base comprises a porous nylon material.

35. A system for microscopic examination of a tissue specimen, comprising:

a porous support to which the tissue specimen adheres in a cytocoherent fashion, and on which the tissue specimen can be lyophilized under conditions that form perfusion pores in the tissue specimen while retaining its cytocoherence; and

a perfusion attachment capable of being secured to the porous support, and perfusing a liquid through the tissue specimen and porous support under pressure without disrupting the cytocoherence of the tissue specimen.

36. The system of claim 35, wherein the perfusion attachment comprises a chamber with an annular seal for placement against the support.

37. The system of claim 35, wherein the porous support comprises a nitrocellulose film adhered to a porous base member that has an optical transmittance that is at least 60% of the optical transmittance of glass.

38. The system of claim 37, wherein the nitrocellulose film is less permeable to flow of liquid than the lyophilized tissue specimen, so that the porous base member limits the flow of the liquid through the tissue specimen.

FIG. 1A

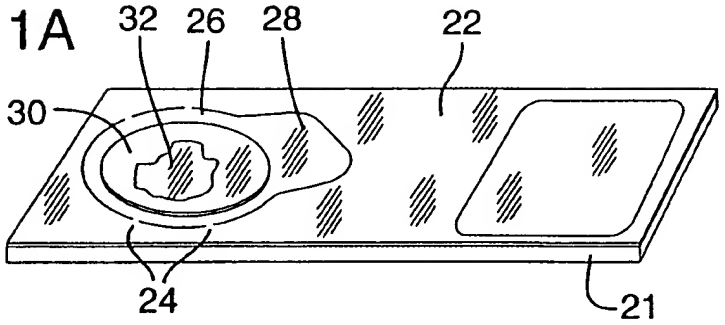


FIG. 1B

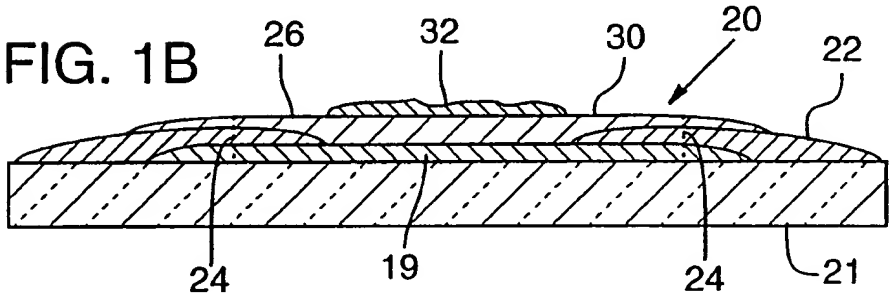


FIG. 1C

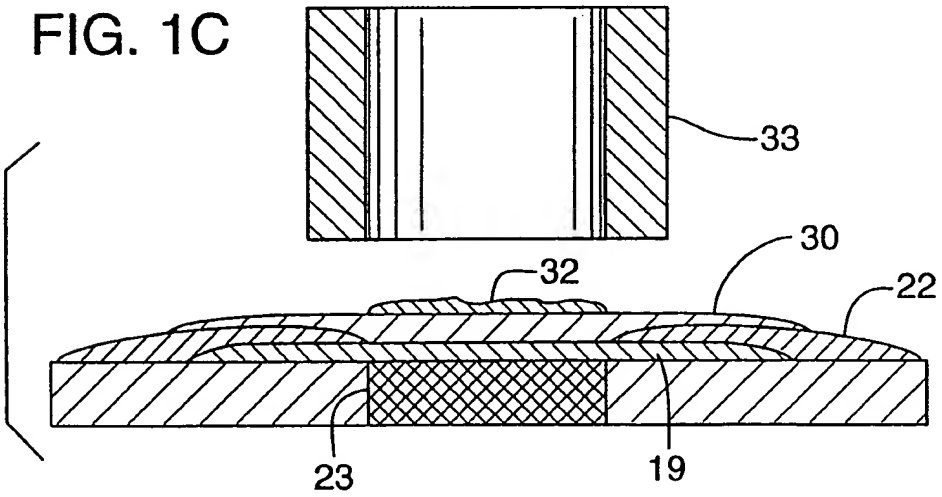


FIG 2

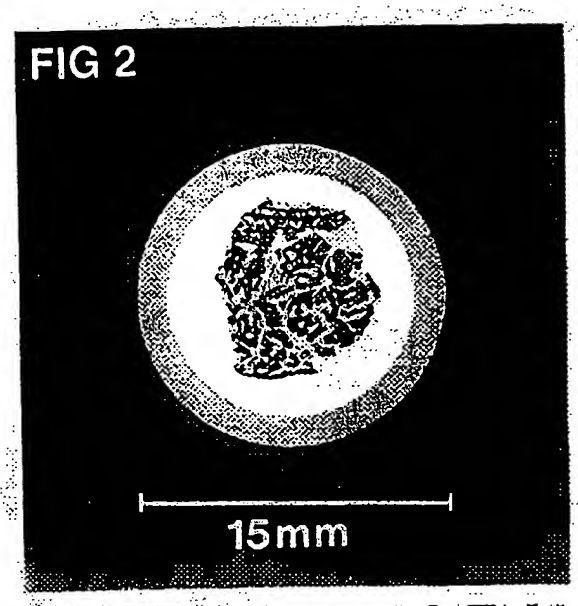


FIG 3

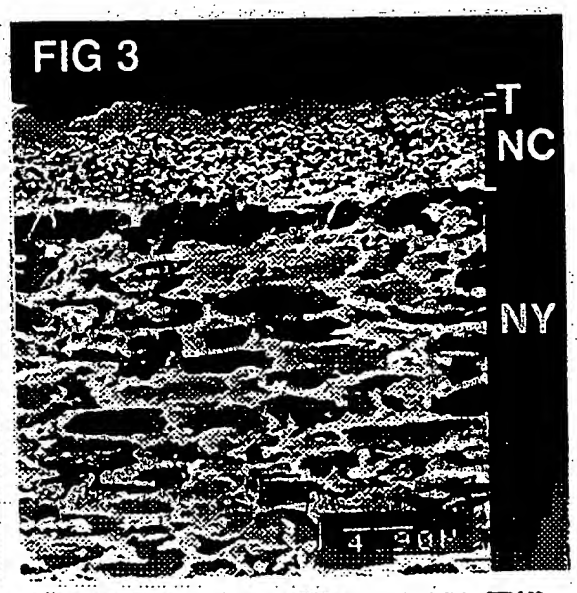


FIG 4

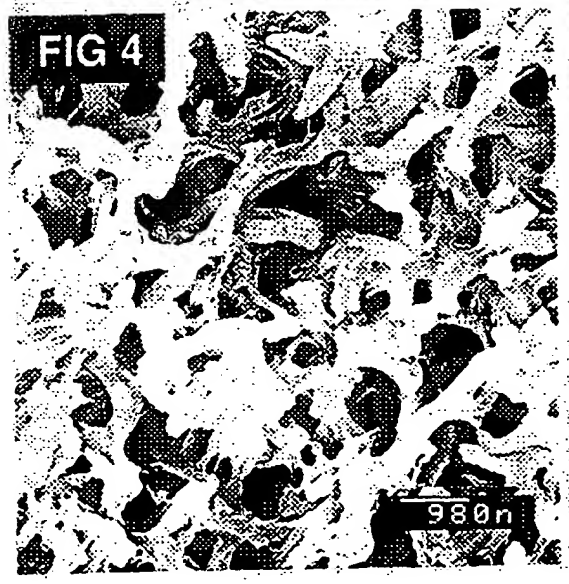


FIG 5

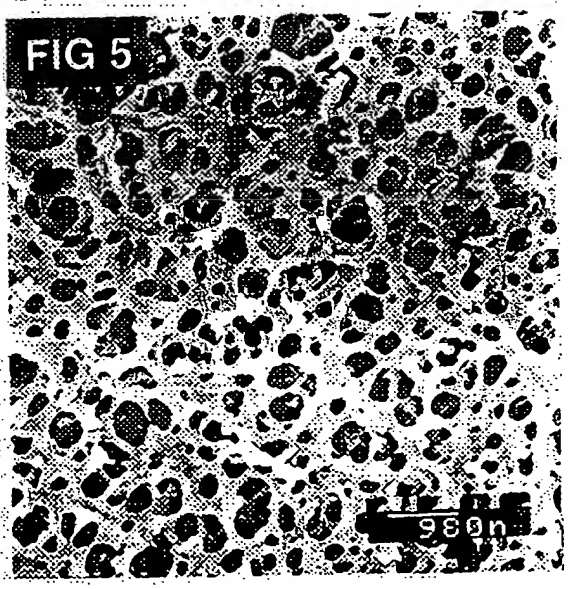


FIG. 6

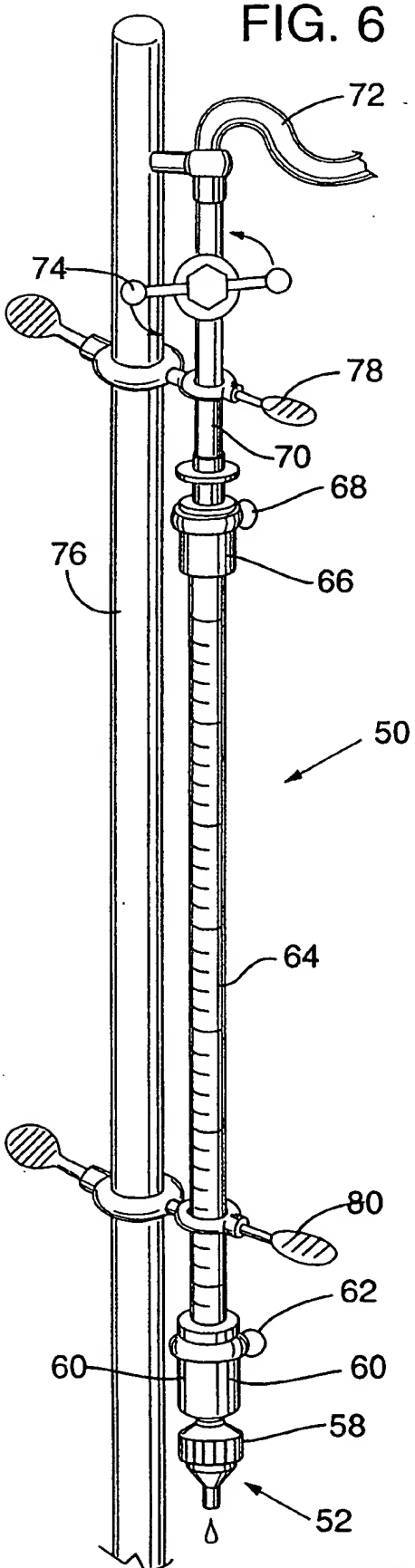


FIG. 7

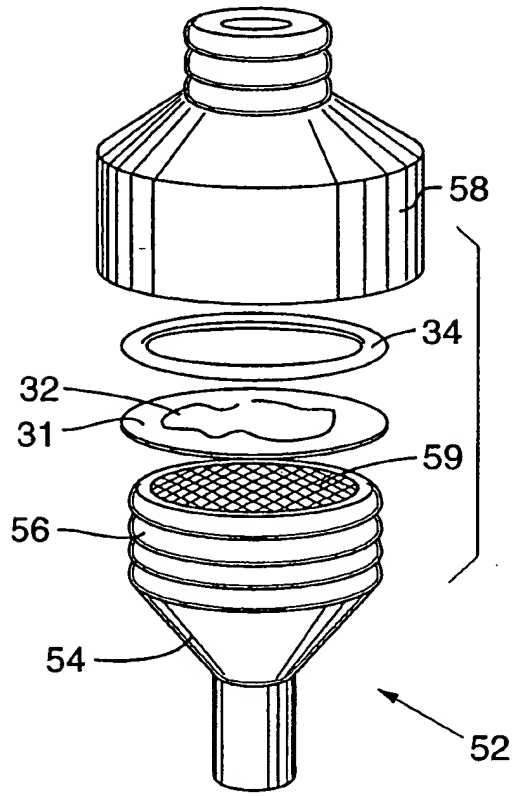
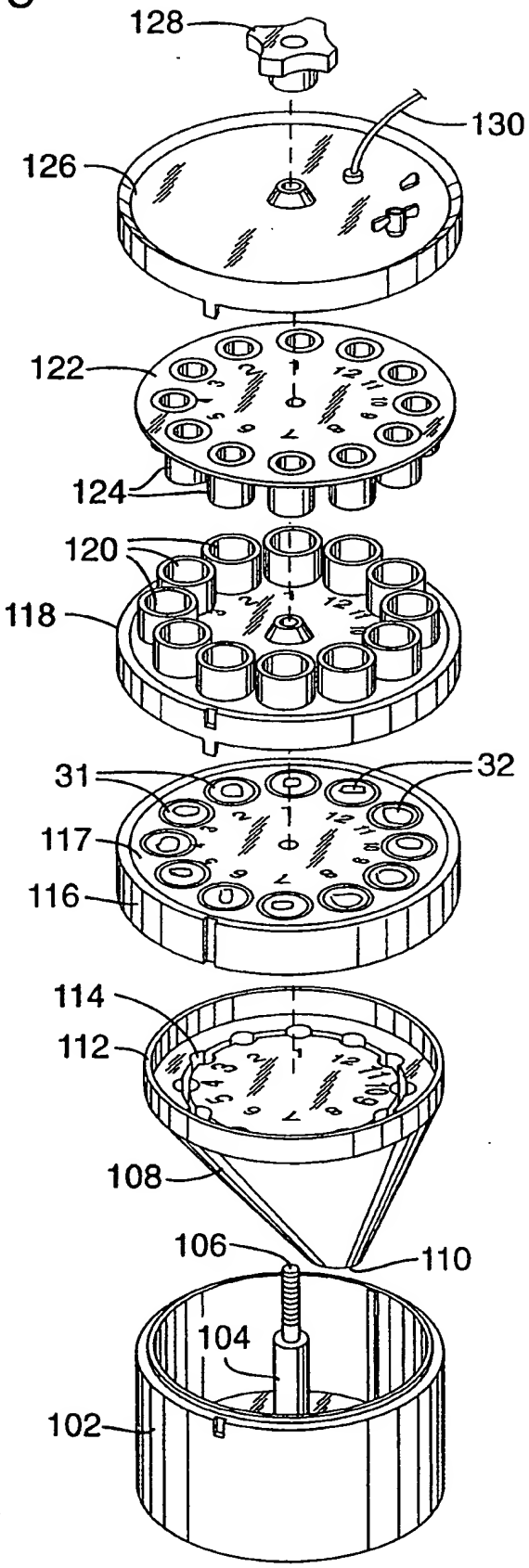


FIG. 8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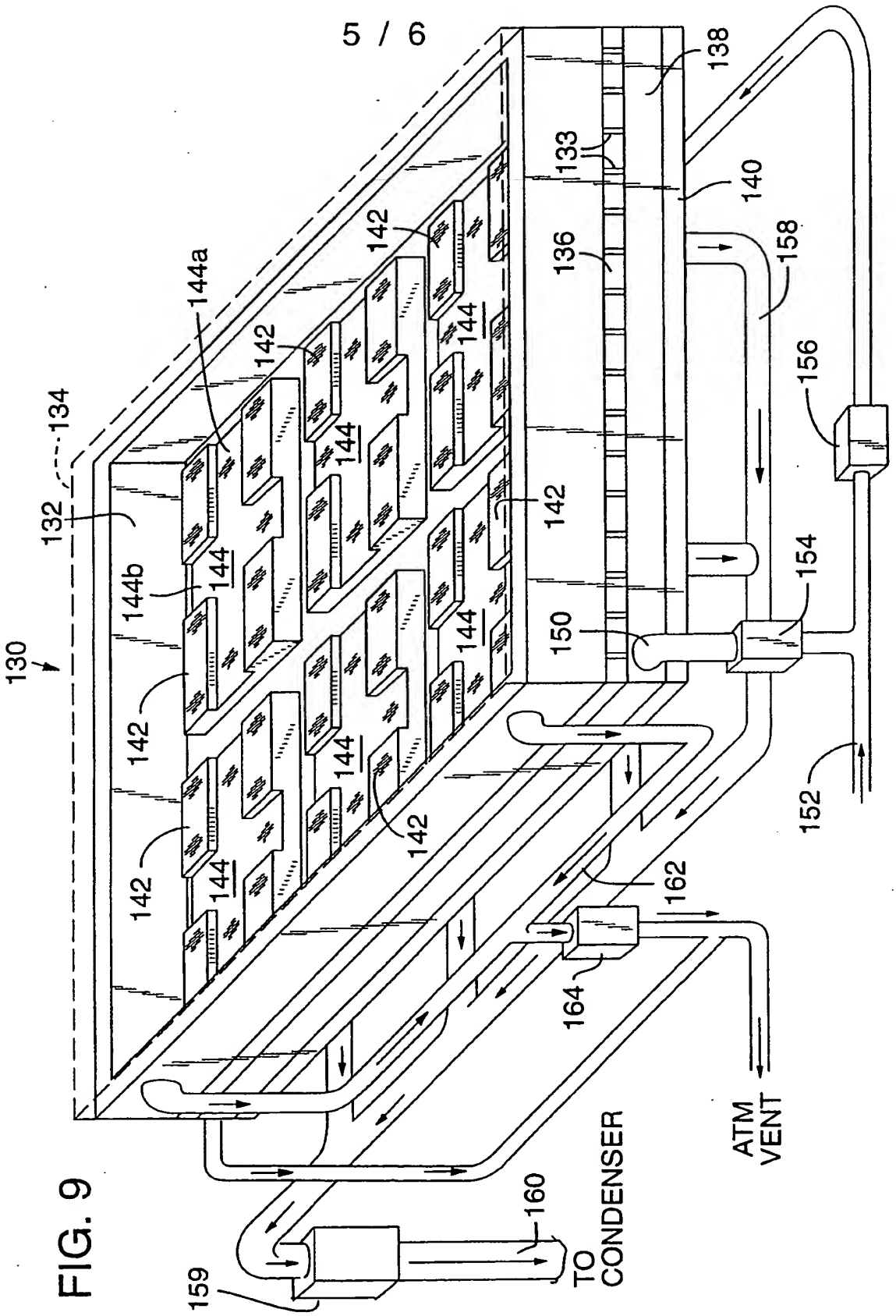
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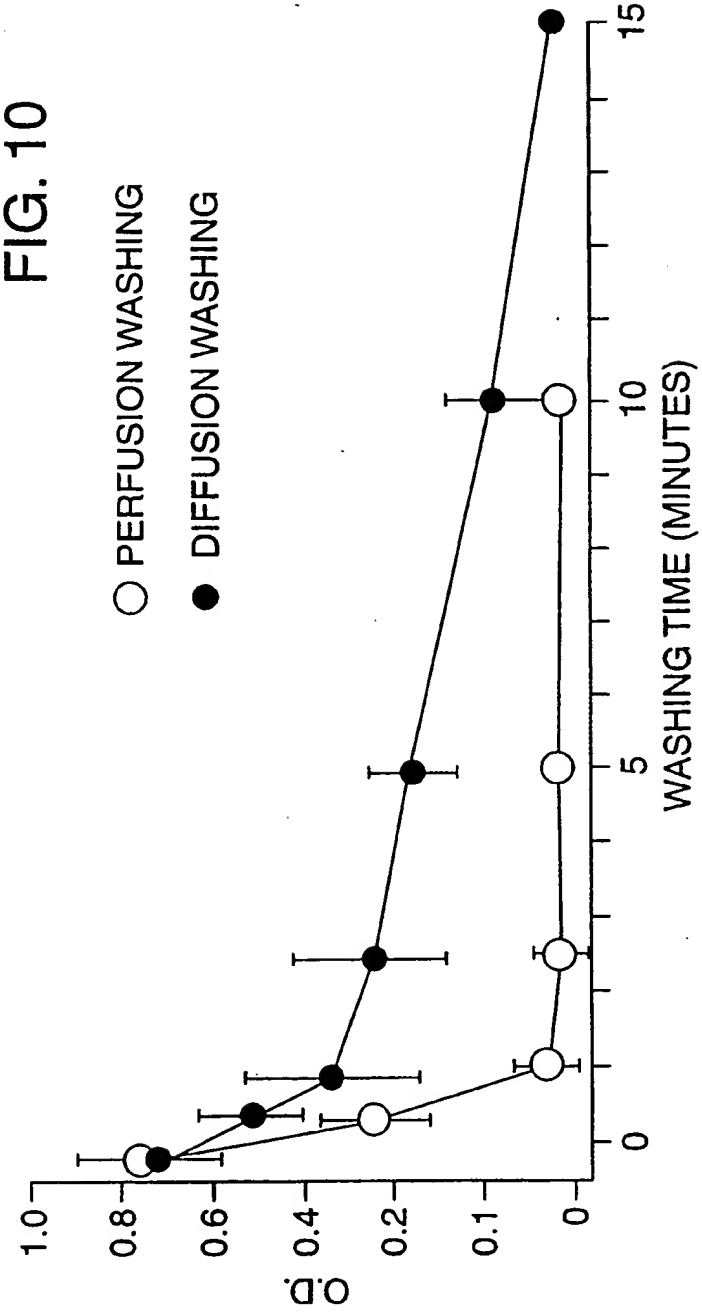
FIG. 9

FIG. 10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● DIFFUSION WASHING
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0.2
0.1
0
0
5
10
15
WASHING TIME (MINUTES)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/19871

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 33/567

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/176, 519; 422/101, 102; 435/7.21, 284.1, 287.2, 287.7, 307.1,

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 93/04193 A (MCGRATH ET AL) 04 March 1993 (04.03.93), see pages 17-20.	1-38
Y	BioTechniques, Volume 11, No. 3, issued 1991, MCGRATH ET AL, "Cytometrically coherent transfer of receptor proteins on microporous membranes", see pages 352-4, 356, 358-61.	1-38
Y	US 4,894,157 A (JOHNSON) 16 January 1990 (16.01.90), see column 1, lines 9 and 38; column 3, lines 1-5 and 39; and column 4, line 7.	9, 10, 18-34, 37-38
Y	US 4,681,853 A (HARDY ET AL) 21 July 1987 (21.07.87), see figure 2.	1-17, 19-20, 23-24, 26-27, 35-38

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"B"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
19 JANUARY 1998	26 FEB 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JAN M. LUDLOW Telephone No. (703) 308-0651

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/19871

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

436/176, 519; 422/101, 102; 435/7.21, 284.1, 287.2, 287.7, 307.1,

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

STN, APS
search terms: histolog? or microscop? or cytolog? or tissue; nitrocellulose; lyophili? or dry### or dried; pore# or porous or porosity or micropor? or channel? or microchannel?; support### or nylon or nytran or s=ceramic or cellulose; detergent or dodecyl or lauryl or sds